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Thermodynamic and Kinetic Study on the Protonation of Free Base Tetraphenylporpyrin Derivatives in Solution

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The protonation of tetraphenylporphyrin (TPP) in acidic organic solutions was analyzed by acid titrimetric and temperature-dependent absorption measurements. Competition between the protonation of free base TPP ($TPPH_2$) and the solvation of proton by near solvent molecules determines the equilibrium of the diprotonated TPP ($TPPH_4^{2+}$) formation. The diprotonated TPP exists as an ion pair complex with the acid counterions, which are found to affect the degree of red shift of the Soret band. The rotation of the phenyl rings also plays an important role in the diprotonation, as suggested by the decrease in the degree of diprotonation for the fluorophenyl TPP derivatives whose phenyl ring rotation is significantly hindered relative to normal TPP. The difference of fluorescence lifetime between $TPPH_2$ (τ_{FL}=19.6 ns) and $TPPH_4^{2+}$ (τ_{FL}=2.1 ns) was used advantageously to measure the rate of protonation in the excited state. The protonation of $TPPH_2$ are found to occur much slower than the diffusion of protons from bulk solution to the porphyrin ring. The monoprotonated TPP is suggested to be the transient species for the diprotonation process.

Introduction

The protonation of free base porphyrins greatly affects their physicochemical properties such as the spectroscopic modifications,[1-5] the behavior of self-association or aggregation,[6-9] and the photoinduced electon transfer to adjacent molecules.[10,11] Upon protonation, the Soret band of porphyrins in UV-vis spectrum is largely shifted to a longer wavelength, which changes the color of porphyrin solutions from the red to the deep green. Also the characteristic Q_x and Q_y bands of the D_{2h} symmetric free base porphyrins are converted to a series of Q bands as a result of the formation of D_{4h} symmetric dicationic forms.[1,2] The protonation also changes the fluorescence properties; fluorescence spectrum of the diprotonated TPP ($TPPH_4^{2+}$) has a broad emission band with the maximum located between the two emssion bands originally observed in the free base TPP ($TPPH_2$).[6] This spectral change has been attributed to the protonation-induced structural change of the porphinato macrocycle.[12-15] Also the rotation of the phenyl rings has been accounted for the variation of the spectroscopic properties in terms of resonance interaction of the phenyl ring with the porphyrin's corresponding orbitals, based on the X-ray crystallographic result which showed that the phenyl substituents reorient from out-of-plane to in-plane position with respect to the porphyrin ring upon protonation.[16,17]

The protonation of free base porphyrins is completely reversible, and the degree of diprotonation at equilibrium is strongly dependent on the type of solvent as well as the acid concentration.[2,18] The effect of acid counterion on the conformational and spectroscopic changes is also menifest in the protonation of various porphyrin derivatives.[4,17] However, the equilibrium thermodynamic properties for the formation of diprotonated porphyrins have been yet rarely studied with respect to any of the environmental factors such as solvent, acid concentration, and the acid counterion effect, not to mention the kinetic property for the protonation process. Therefore, we investigated the equilibrium thermodynamic and kinetic properties for the protonation of tetraphenyl porphyrin and its fluorophenyl derivatives in organic solvents using acid titrimetric absorption and fluorescence lifetime measuring techniques. Our report will demonstrate that the formation of the diprotonated TPP goes through an activation state in the form of the monoprotonated TPP, and the equilibrium properties for the diprotonation are characterized by the proton exchange between

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the free base porphyrin ring and the adjacent solvent molecules. The stability of the diprotonated porphyrins is found to be closely related to the steric interaction between the porphyrin ring and the phenyl substituents as revealed by a series of experiments which shows that the dependence of the degree of diprotonation at equilibrium is strongly dependent on the positions of fluorine atoms on the phenyl ring.

Experimental

$TPPH_2$ was synthesized by the well estabilished method by condensation of pyrrole and benzaldehyde.[19] Tetrakis(2-fluorophenyl) porphyrin ($2FTPPH_2$), tetrakis(3-fluorophenyl) porphyrin ($3FTPPH_2$) tetrakis(4-fluorophenyl) porphyrin ($4FTPPH_2$), and tetrakis(2,6-difluorophenyl) porphyrin ($6FTPPH_2$) were synthesized by the modified Rothemund method starting from the corresponding fluoro derivatives of benzaldehyde.[20,21] All products were purified by chromatography on silica gel with methylene dichloride and recrystallized.

All solvents in this study were purchased as EP grades from Aldrich and used as received. In the open air, $TPPH_2$ and its derivatives have a tendency to be slowly oxidized in organic solvents resulting in spectral broadening, so all TPP solutions were made and stored tightly sealed, which were used in less than a few days for spectroscopic measurements. Absorption spectra were obtained by either a Shimadzu UV-2401PC Spectrophotometer or a HP-8453 Spectrophotometer with a 10 mm path length cell located in the temperature controlled cell adaptor. Stationary fluorescence spectra were taken with an ISS Spectrofluorometer and fluorescence lifetimes were measured by a PTI TimeMaster with 0.1 ns time resolution. The instrumental response of the lifetime measurement was checked by observing the scattered light from an aqueous colloidal silica solution. Fluorescence lifetimes were measured through an OG-550 cut-off filter with the 5 nm bandwidth excitation.

Results and Discussion

Equilibrium of the $TPPH_4^{2+}$ Formation. Figure 1 shows the absorption change of the $TPPH_2$/DMSO solution by titmetric addition of HCl. Upon the addition of HCl, a new absorption band appears at 448 nm and its intensity increases at the expense of the decrease of the original Soret band at 418 nm. In addition to the red shift of the Soret band, the formation of diprotonated TPP is also evident in the result that the four Q bands characteristic of the D_{2h} symmetric $TPPH_2$ diminish altogether while a rather strong band appears at 665 nm with its much weaker vibrational analogs, which is typical of the D_{4h} symmetric $TPPH_4^{2+}$. At any acid concentration, no other band except the mentioned ones appeared, implying that the concentration of the monoprotonated $TPPH_3^+$ is, if any, too low to be detected in the steady state absorption spectra. If we neglect the ion pairing effect of the acid counterion, the protonation of $TPPH_2$ can be represented by

$$TPPH_2 + 2H^+ \rightleftarrows TPPH_4^{2+} \quad (1)$$

where the equilibrium constant for the diprotonation, K_D, is

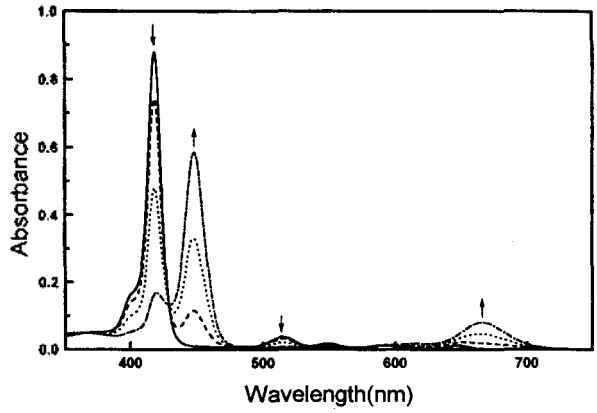

Figure 1. HCl titmetric absorption spectra of 2.3×10^{-6} M $TPPH_2$ in DMSO. From the top of the 418 nm band, [HCl] were 0 (solid), 5.0×10^{-3} (dashed), 1.1×10^{-2} (dotted), 2.2×10^{-2} M (dot-and-dashed), respectively.

given by

$$K_D = \frac{[TPPH_4^{2+}]}{[TPPH_2]\,[H^+]^2}. \quad (2)$$

Given the experimental condition that the acid concentration is much greater than the initial TPP concentration, the equilibrium between the two forms can be denoted by the partial fraction of the diprotonic form (f_D) as a function of the acid concentration, assuming a complete dissociation of the acid in the solution.

$$f_D = \frac{K_D \times [\text{acid}]^2}{1 + K_D \times [\text{acid}]^2} \quad (3)$$

The measured f_D values are plotted in Figure 2 as a function of [HCl] with a model fit given by Eq. (3). The possiblility for the formation of sizable amount of $TPPH_3^+$ is well ruled out because of the large discrepancy between the experimental data and the model fit using 1 for the exponent of [HCl]. The best fit is obtained by taking between 2 and 3 for the exponent of [HCl]. The fact that the exponent of [HCl] is greater than 2 implies that the dipro-

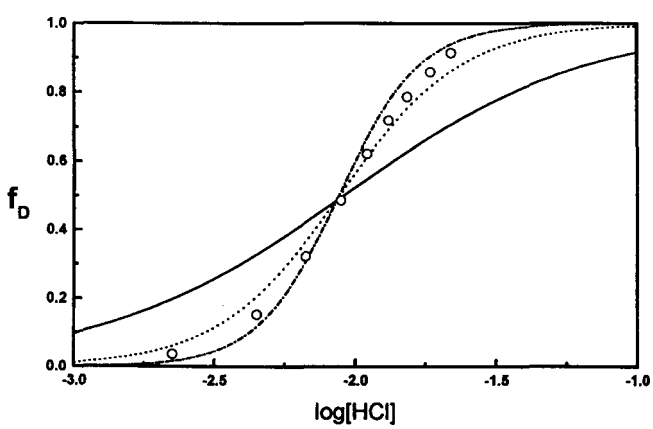

Figure 2. Degree of formation of $TPPH_4^{2+}$ in DMSO as a function of [HCl] at 300 K. Open circles represent the experimental data while the solid, dashed, and dot-and-dashed lines represent the model calulation with 1, 2, and 3, repectively, for the exponent of [HCl].

tonation process is at least partially affected by the binding of acid counterions.

Environmental Effects. The effect of the acid counterion is evident from the result that the degree of red shift varies depending on the type of acid counterion; in acidic DMSO, the red shift was 1700 cm^{-1} for HCl, 1280 cm^{-1} for H_2SO_4, 1440 cm^{-1} for $HClO_4$, and 1440 cm^{-1} for HNO_3. The paired anionic counterion is considered to lower the upper energy levels by enhancing the charge delocalization, in which the bulkier counterions are found to be not so effective as the chloride ion in lowering the excited orbital energy level. The counterion effect was further verified by an experiment in which the addition of a large amount of $Bu_4N^+ClO_4$ into the H_2SO_4- and HCl-added $TPPH_2$/DMSO solutions changed the value of red shift from 1280 to 1440 cm^{-1}, and 1700 to 1440 cm^{-1}, respectively. It is also to be noted that the degree of diprotonation is dependent on the type of the acid counterion in the decreasing order of $Cl^- > ClO_4^- > SO_4^{2-} > NO_3^-$ at a given acid concentration.

The solvent effect was also remarkable, leading to the big difference in the degree of diprotonation at equilibrium between the DMSO and acetone solutions; compared with the solution of $TPPH_2$ in DMSO, the acid concentration required to get the half conversion decreased by a factor of 100 in acetone solution. Considering that the dielectric constant of DMSO (ε=46.7) is much larger than that of acetone (ε=20.7), the solvation of proton by solvent molecules is thought to compete with the protonation of the porphyrins. It was clearly demonstrated by an experiment which showed that the dropwise addition of water (ε=78.5) into the $TPPH_2$ in acidic acetone solution monotonically decreased the degree of protonation.

The dependence of K_D on the initial concentration of $TPPH_2$ was measured to check whether the aggregation of the protonated TPP was involved in the acid titrimetric experiment or not. The increase of the $TPPH_2$ concentration by two times hardly affected the ratio of the two forms, indicating that the dimer-like formation between porphyrin molecules is not involved in the protonation process, which is consistent with the Raman spectroscopic result that the TPP derivatives having neutral substituents hardly form aggregates in acidic organic solution.[6]

Effect of Phenyl Groups on the Diprotonation. Absorption profiles of the free base and diprotonated forms of all fluoro derivatives of $TPPH_2$ were found to be very similar to those of normal TPP. However, the K_D values differed markedly depending on the position of fluoro substituents on the phenyl ring. Figure 3 illustrates the degree of diprotonation as a function of HCl concentration for the various TPP derivatives in DMSO. The lines represent the model fit of Eq. (3) with 2 for the exponent of [HCl]; the K_D values were calculated to be 1.0×10^4 ($TPPH_2$), 7.0×10^3 ($4FTPPH_2$), 1.5×10^3 ($3FTPPH_2$), 10 ($2FTPPH_2$), and 0 ($2,6FTPPH_2$). The closer the fluorine substituents are located to the internal porphyrin ring, the smaller the K_D values result. One of the reasons why the fluorophenyl derivaties are hard to be protonated is probably the steric effect of the fluorophenyl substituents on the protonated porphyrin ring whose conformation is significantly distorted compared to the near-planar free base porphyrin ring. Since the diprotonated porphyrin is more stable with its phenyl substituents

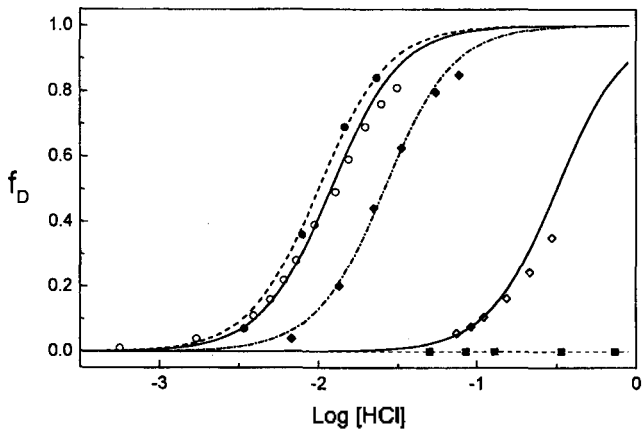

Figure 3. The degree of diprotonation as a function of HCl concentration for the various TPP derivatives in DMSO; closed circle ($TPPH_2$), open circle ($4FTPPH_2$), closed diamond ($3FTPPH_2$), open diamond ($2FTPPH_2$), and closed square ($2,6FTPPH_2$). The lines represent the model fit with 2 for the exponent of [HCl].

positioned parallel to the porphyrin internal ring, while the free base porphyrin is more stable with the phenyl substituents in perpendicular position,[16] the energy increase during the phenyl rotation for the fluorophenyl porphyrins must be accounted for the protonation kinetics.[17] The electron withdrawing effect of the fluorine atoms on the phenyl rings might also prevent the diprotonation by reducing the electron density of the porphyrin internal ring, even though this effect is expected to be much smaller than the steric effect as shown in the result that p-fluorophenyl TPP has the higher K_D value than those of both m-fluorophenyl and o-fluorophenyl TPP's.

The enthalpy changes in the formation of diprotonated TPP derivatives were checked by the temperature dependent absorption change. For $TPPH_2$ in acidic DMSO (1.2×10^{-2} M), the temperature increase resulted in the change of the original and red shifted Soret band intensities. [Figure 4] The ratio of the two band intensities was recovered when the temperature was decreased backwards. To analyze the effect of fluorine atoms on the protonation, thermodynamic data of the diprotonation were obtained by measuring the temperature dependent absorption spectra of the $TPPH_2$, $2FTPPH_2$, and $2,6FTPPH_2$ (each 3×10^{-6} M) in ethylene glycol solutions with 4.0×10^{-5}, 1.0×10^{-3}, and 3.0×10^{-3} M [HCl], respectively. The acid concentrations were adjusted to get ~70% protonation conversion for each TPP derivatives. All solutions showed monotonic decrease of f_D as the temperature increased, which is very similar to the result of $TPPH_2$ in acidic DMSO solution. The Van't Hoff plots show that the ΔH values for the diprotonation of $TPPH_2$, $2FTPPH_2$, and $2,6FTPPH_2$ are -120, -79, and -53 kJ mol^{-1}, respectively, based on the KD calculation with 2 for the exponent of [HCl]. [Figure 5] The reduction of the diprotonation enthalpies for the fluorinated TPP's may be attributed to the increase of the steric energy between the diprotonated porphyrin ring and the fluorine containing phenyl substituents, as discussed in the acid titrimetric experiment.

The result that the measured ΔH values are significantly smaller than the usual proton binding energy may be at-

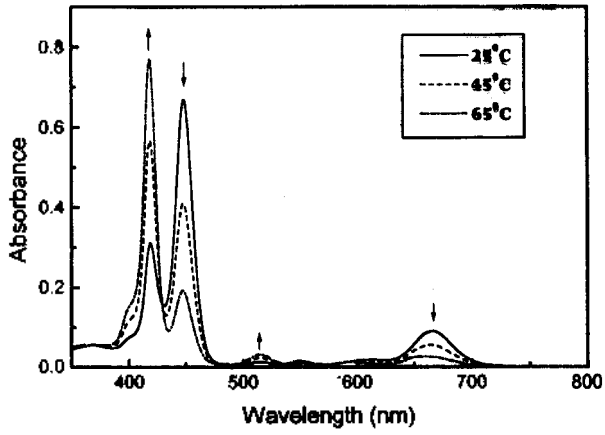

Figure 4. Temperature dependent change of the absorption profile of $TPPH_2$ in acidic DMSO (1.2×10^{-2} M HCl).

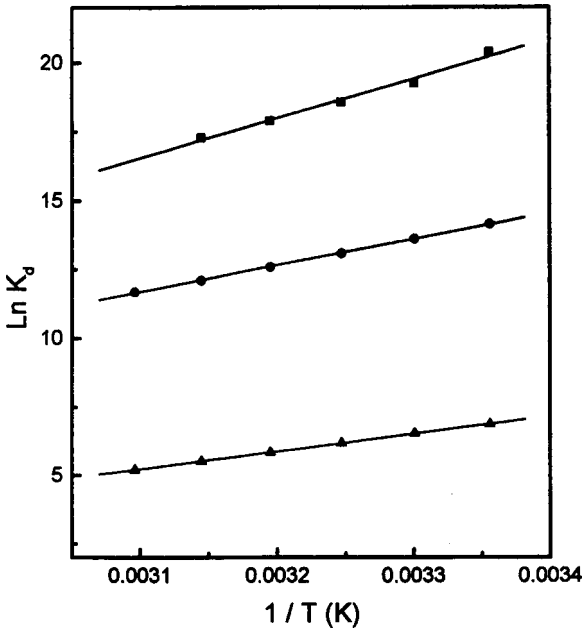

Figure 5. Van't Hoff plots of $TPPH_2$ (square), $2FTPPH_2$ (circle), and $2,6FTPPH_2$ (triangle) in acidic ethylene glycol.

tributed to the loss of the proton solvation energy during the diprotonation process. For $TPPH_2$, the ΔH value of the diprotonation in acidic DMSO solution (-54 kJ mol^{-1}) was found to be reduced by half compared with that in the acidic ethylene glycol solution, clearly supporting the above argument. However, the difference in ΔH between these two solvents would not be well explained by considering only the proton solvation effect. Additional environmental factors related to the counterion pair formation should be also considered for the full explanation of the solvent effect.

Fluorescence Studies. In DMSO, the fluorescence profile of $TPPH_2$ has two Q(0,0) and Q(0,1) bands at 651 and 715 nm, respectively, whereas only one broad emission band appears at 693 nm for $TPPH_4^{2+}$. It is noteworthy that the Stokes shift of $TPPH_4^{2+}$ ($\Delta\upsilon$=608 cm^{-1}) is significantly greater than that of $TPPH_2$ ($\Delta\upsilon$=95 cm^{-1}). [Figure 6] The increase of the Stokes shift upon the diprotonation is considered to be due to the formation of the $TPPH_4^{2+}$-counterion pair having large dipole moment, which requires a large extent of solvent reorientation for the dielectric solvation of photoexcited $TPPH_4^{2+}$.[23]

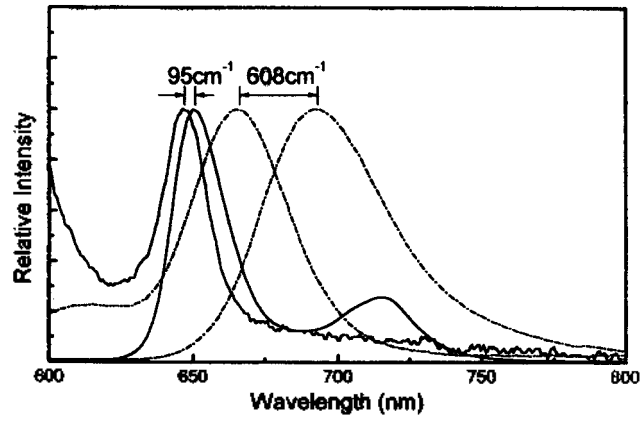

Figure 6. Absorption and fluorescence spectra of $TPPH_2$ (solid curves) and $TPPH_4^{2+}$ (dashed curves) in DMSO, showing the dramatic increase of Stokes shift upon protonation. Fluorescence excitation wavelengths for $TPPH_2$ and $TPPH_4^{2+}$ are 418 and 448 nm, respectively.

The fluorescence lifetime (τ_{FL}) of $TPPH_2$ is also dramatically affected by the protonation. The deconvoluted lifetime of $TPPH_4^{2+}$ was measured 2.1 ns compared with 19.6 ns of $TPPH_2$. [Figure 7B and 7C] With respect to $TPPH_2$, the reduction in the fluorescence lifetime for $TPPH_4^{2+}$ has been explained by the protonation-induced perturbation of either S_0 or S_1 state, which results in the enhancement of the nonradiative decay processes such as intersystem crossing from S_1 to T_1, internal conversion from S_1 to S_0, and intramolecular charge transfer.[24-26]

The difference in fluorescence lifetime between the two forms was advantageously employed to study the dynamics of protonation. When the free base and diprotonated forms coexist in the solution of 1×10^{-7} M $TPPH_2$ in acidic DMSO (1×10^{-2} M HCl), the fluorescence lifetime of each form would be affected by the protonation and deprotonation-related relaxation processes, respectively. Excitation at 418 nm (absorption peak of free base form) showed a noticeable reduction of the fluorescence decay time compared with that of the $TPPH_2$-only solution, while excitation at 448 nm (absorption peak of diprotonated form) yielded almost the same result as the $TPPH_4^{2+}$-only solution [Figure 7a and 7b]. At first glance, the reduction of the fluorescence lifetime excited at 418 nm looks as if it is related to the protonation process which is expected to convert the slow-decaying excited $TPPH_2$ into the fast-decaying excited $TPPH_4^{2+}$. However, it must be noted that the $TPPH_4^{2+}$ contribution at 418 nm *via* the excitation of B(1,0) band must be subtracted out from the fluorescence decay profile of Figure 7b. A judicious subtraction of that portion from the raw data showed that fluorescence of $TPPH_2$ in acidic solution decays in the same rate as that of $TPPH_2$ in non-acidic condition. So the result confirmed that the protonation of the photoexcited $TPPH_2$ in acidic DMSO occurs significantly slower than the fluorescence lifetime of $TPPH_2$ (19.6 ns), which implies that the protonation of photoexcited $TPPH_2$ in acidic DMSO solution occurs much slower than the dif-

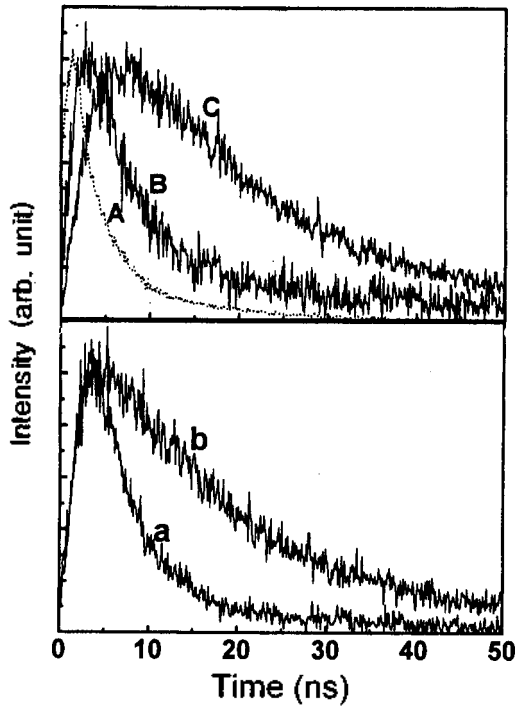

Figure 7. Top: fluorescence decay profiles of the lamp (A), $TPPH_2$ in DMSO excited at 418 nm (B), $TPPH_4^{2+}$ in acidic DMSO (5×10^{-2} M HCl) excited at 448 nm (C). Bottom: fluorescence decay profiles of $TPPH_2$ (excited at 418 nm) and $TPPH_4^{2+}$ (excited at 448 nm), which coexist in DMSO solution with 1.0×10^{-2} M HCl. Decay signals are collected at all wavelengths through an OG-550 filter.

fusion controlled rate.

Considering the fact that the monoprotonated form was not detected in a sizable amount at any acid concentration, it is reasonable to attribute $TPPH_3^+$ to the transient species in the diprotonation process of $TPPH_2$. An X-ray structure study showed that the monoprotonated porphyrin ring has a severely distorted conformation, underlining that the monoprotonated form is much more unstable than both the free base and diprotonated ones.[16] For $TPPH_2$, steric effects exerted by the meso phenyl groups may further destabilize the monoprotonated species. It is to be reminded that the monoprotonated species can exist as a stable form for the unsubstituted or alkyl chain-substituted porphyrin compounds in acidic organic solution.[22] Therefore, for TPP derivatives, the rotation of phenyl rings from the out-of-plane to in-plane geometry may assist in the monoprotonation process by reducing the activation energy to form the transient $TPPH_3^+$. However, with the given experimental results, it is not clear whether the phenyl ring rotation in the diprotonation occurs before or after the formation of $TPPH_3^+$.

Summary

The formation of the diprotonated TPP derivatives in acidic organic solutions was analyzed by acid titrimetric and temperature dependent absorption measurements. In DMSO, the competetion between the protonation of free base TPP and the solvation of proton by near solvent molecules determines the equilibrium of the diprotonation. Acid titrimetric absorption change of the TPP derivatives in DMSO solutions is well fit to an equilibrium model calculation which is based on an assumption that the diprotonation process is dominated by the binding of two protons to the free base porphyrin ring. The diprotonated TPP is found to exist as an ion pair complex with the acid counterions as confirmed by the variation both in the degree of the Soret band's red shift and in the degree of diprotonation with respect to the type of acid counterion. The steric effect exerted by the phenyl rings also plays an important role in the diprotonation, as suggested by the result that the degree of diprotonation for TPP derivatives is found to be largely affected by the positions of the fluorine atoms on the phenyl ring.

In this report, the fluorescence lifetime measurement on $TPPH_2$ (τ_{FL}=19.6 ns) and $TPPH_4^{2+}$ (τ_{FL}=2.1 ns) was employed to measure the rate of protonation. The protonation of $TPPH_2$ in the excited state was found to occur much slower than the fluorescence lifetime, which means that it occurs much slower than the diffusion rate of protons from bulk solution phase to the porphyrin ring. The experimental result that the diprotonation is not entirely diffusion controlled implies the existence of a transient species, which needs the activation energy to undergo the diprotonation reaction. Given the fact that the monoprotonated form was not observed in appreciable amount at any acid concentration in the titrimetric absorption experiments, $TPPH_3^+$ is suggested to be the transient species for the diprotonation process.

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Effect of Ethidium on the Formation of Poly(dA)·[poly(dT)]$_2$ Triplex: A Kinetic Study by Optical Spectroscopic Methods

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The kinetics of the formation of triplex poly(dA)·[poly(dT)]$_2$ from poly(dA)·poly(dT) and poly(dT) is examined by various optical spectroscopic methods, including absorption, circular and linear dichroism (LD) spectroscopy. In the pseudo first order condition, where the poly(dT) concentration is kept lower than that of duplex, the association of the poly(dT) is enhanced by the presence of ethidium; the rate constant is proportional to the amount of ethidium in the mixture. When the concentration of the duplex and the single strand is the same, a spectral change is explained by double exponential curves, indicating that at least two steps are involved, the fast association and slow rearrangement steps. In contrast to the pseudo first order kinetics, the association step in an equimolar condition is not affected by the presence of ethidium. In the rearrangement step, the magnitude of LD decreases with an increase in ethidium concentration, suggesting that the bending of polynucleotide around the intercalation site occurs in the rearrangement step.

Introduction

Pyrimidine and purine oligonucleotides can bind to the major groove of duplex DNA at a specific homopurine·homopyrimidine sequence to form triplex DNA. The high selectivity of the third strand makes it an effective method for recognizing a single site in a large duplex DNA. This concept leads to potential biological and therapeutic applications, such as inhibiting gene expression and to designing artificial nucleases.[1] Thus, it is important to investigate the kinetic aspect of the interaction between a homopurine·homopyrimidine duplex DNA and a single stranded DNA which form a triplex. Indeed, there have been several studies, using different techniques, of the kinetics of triplex formation. The techniques included a restriction endonuclease (*AvaI*) protection assay,[2] DNase I footprining,[3] filter binding assay,[3,4] fluorescence resonance energy transfer,[4] UV absorption techniques[5,6] and biomolecular interaction analysis.[7] The association rate constant (k_1) between the oligonucleotides and the target duplex was reported to be 10^3 $M^{-1}s^{-1}$.

We studied kinetics of triplex formation between poly(dT) and poly(dA)·poly(dT) using absorption, fluorescence, and polarized light spectroscopy namely, circular dichroism (CD) and linear dichroism (LD). Although a polynucleotide does not possess any chiral center, it acquires an induced CD spectrum from the excitonic interactions between the electric transition moment of the nucleo-bases.[8] Therefore, CD mainly examines the changes in nucleo-base stacking during triplex formation. The polynucleotide conformation can be examined from the CD of intrinsic polynucleotide absorp-

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